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Continuous, On-Line Interface for Reverse-Phase Microbore High-Performance Liquid Chromatography/Diffuse Reflectance Infrared Fourier Transform Analysis

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Continuous, On-Line Interface for Reverse-Phase Microbore High-Performance Liquid Chromatography/Diffuse Reflectance Infrared Fourier Transform Analysis*

M. A. CASTLES, L. V. AZARRAGA,† and L. A. CARREIRA

U.S. Environmental Protection Agency, Environmental Research Laboratory, Athens, Georgia 30613 (M.A.C., L.V.A.); and University of Georgia, Department of Chemistry, Athens, Georgia 30602 (M.A.C., L.A.C.)

An ultrasonic nebulizer with a piezo-electric transducer and a newly designed and constructed continuous on-line collection station have been adapted as an interface between a reverse-phase microbore high-performance liquid chromatograph (RP-μHPLC) and a diffuse reflectance infrared Fourier transform spectrometer. The collection station incorporates a directional vacuum port for confinement of the nebulized material onto a small area (3 to 5 mm i.d.) of the diamond powder. The inherent desolvation that occurs upon nebulization of the reverse-phase eluant, along with the vacuum collection system and the diamond powder diffuse reflectance substrate, makes possible the continuous on-line collection and FT-IR analysis of the compounds separated by a RP-μHPLC without any type of initial solute extraction.

Index Headings: **HPLC/FT-IR, Infrared diffuse reflectance.**

INTRODUCTION

Modern high-performance liquid chromatography (HPLC) has become a powerful technique for separating mixtures into their components, especially for highly polar, nonvolatile compounds. As a general rule, however, commercially available conventional detection systems (e.g., UV/VIS, refractive index, etc.) lack the ability to yield structural information about the compounds eluted from an HPLC column. Because of some shortcomings of these conventional detection systems, Fourier transform infrared spectrometry is one of the novel detection systems that have been used to obtain the much desired structural information.[1]

Many interfaces have been successfully employed for the infrared detection and identification of compounds separated by an HPLC with a 4.6-mm-i.d. column. These interfaces have essentially fallen into two distinct categories, one being the direct flow cell technique[2-4] and the other being the diffuse reflectance-solvent elimination technique.[5,6] Both of these techniques, however, suffer as a result of the high flow rates required for the 4.6-mm-i.d. columns and the low solute-to-solvent ratios.

To circumvent these problems, microbore-HPLC (μHPLC) systems have been implemented. Microbore systems offer much lower flow rates (10 to 100 μL/min) and approximately a 100-fold increase in solute-to-solvent ratio. Several of these systems have already been designed, including a diffuse reflectance-solvent elimination technique in which the eluant is deposited dropwise on a powdered KCl substrate,[7,8] and a buffer memory technique[9,10] in which the normal phase eluant is continuously deposited on a KBr plate. From the KBr plate, the transmission spectra of the separated compounds are recorded. The intrinsic problem with both of these techniques is that they cannot tolerate the aqueous mobile phases used in reversed-phase chromatography.

Diamond powder has proven useful in obtaining diffuse reflectance spectra of compounds initially in aqueous solvents.[11] The use of diamond powder produced several problems, however, that had to be addressed before it could be used as a substrate for sample deposition of the compounds separated by a reverse-phase (RP)-μHPLC.

The major difficulty associated with the use of diamond powder was the problem of depositing the sample(s) on the substrate without disturbing the surface of the powder. Because of its extreme hardness, diamond powder lacks the cohesiveness of KCl; consequently, the particles are loosely packed. Therefore, when the sample was deposited on the surface in drops, the momentum of the drop was sufficient to cause reorientation of the diamond grit.

This reorientation of the diffuse reflectance matrix resulted in a poor background compensation of the spectra taken before and after sample deposition. This poor background compensation resulted in a rolling baseline and spurious peaks throughout the spectrum, thus making it impossible to distinguish the absorption bands from those resulting from the disturbance of the diamond powder.

To circumvent this problem, a low vacuum was applied across the powder, and an ultrasonic nebulizer with a piezo-electric transducer was employed. The first account of its use in HPLC/FT-IR as a sample deposition technique for diffuse reflectance studies was by Azarraga.[12]

In this paper, we present the use of the newly designed collection station and the ultrasonic nebulization technique for the on-line FT-IR analysis of a test solution of *m*-nitroaniline and 2-methyl-3-nitrophenol.

EXPERIMENTAL

Chromatography. The microbore separations of the test compounds were accomplished with a 1-mm-i.d. × 250-mm-long, stainless-steel microbore reverse-phase column (Alltech Associates). The column was packed with 5-μm silica particles. The injector used was a Rheodyne Model 7520 with a 1-μL injection loop. The pump

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† Author to whom correpondence should be sent.

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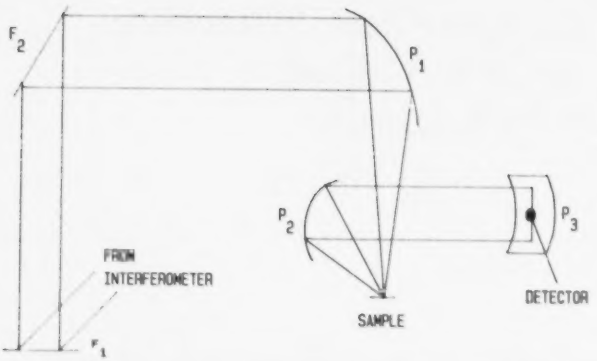

FIG. 1. Schematic diagram of DRIFTS optical configuration. Mirrors F_1 and F_2 are flat plane mirrors used to elevate and steer the infrared beam exiting the interferometer. P_1 and P_2 are focusing and collecting paraboloids, respectively. P_3 is a paraboloid used for focusing the collected infrared radiation onto the detector. Note: Detector is not coplanar with other optics.

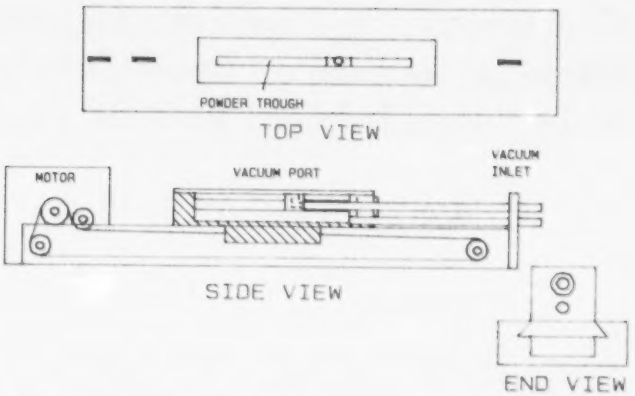

FIG. 2. Schematic drawing of the continuous on-line collection station.

and pressure controllers were Gilson Modeis 302 and 802, respectively. A Kratos Spectroflow Model 773 variable-wavelength UV/VIS detector equipped with a 0.5-μL flow cell was used for the uv detection (254 nm) of the separated compounds. The solvent composition for the separation was 80% methanol and 20% water. A mobile phase flow rate of 40 μL/min was used for the separations.

Optical Configuration. The optical configuration constructed to accommodate the continuous collection system is shown in Fig. 1. The infrared radiation was obtained from a globar source and was directed and collimated with the conventional Digilab source optics. The optical path of the beam from the Michelson interferometer was redirected by means of the two plane mirrors, F_1 and F_2, so that the beam's horizontal axis lies 15 cm above the top of the optical table. The beam was then focused perpendicularly onto the surface of the diamond powder with a 10-cm-focal-length, 90° off-axis paraboloid mirror (P_1), obtained from Barnes Analytical Spectra-Tech. The normal angle of incidence ensures a minimum specular component of the reflectance flux. The diffusely reflected radiation was collected with a 4-cm-focal-length, 60° off-axis paraboloid mirror (P_2). The collimated beam from P_2 was focused by a 4-cm-focal length, 90° off-axis paraboloid (P_3) onto the 2-mm × 2-mm crystal of an MCT detector.

Spectrometry. The FT-IR measurements were obtained with a Digilab 296 interferometer, removed, along with the accompanying electronics and source optics, from a Digilab Model FTS-14 c/x cabinet. The interferometer and source were mounted on an optical table to facilitate operation of the diffuse reflectance optical system and the continuous collection station. A medium-range MCT equipped with a 2-mm-square crystal (Cryogenic Association, Inc.) was used for all measurements. All spectra were recorded at a resolution of 8 cm^{-1}. The interferograms collected under the control of the GIFTS software package were single scan, single precision.

Digilab IMX software was used for collecting the double-precision, signal-averaged spectra. The Gram-Schmidt (G.S.) orthogonalization algorithm was used to reconstruct the chromatogram as the separated components were deposited on the substrate. The interferograms that were used to compute each of the sample spectra shown in this paper were taken from an area surrounding the maximum of each peak in the reconstruction. Because the maximum corresponded to those interferograms that represented the greatest amount of infrared absorption by the compound, the spectra of greatest absorption intensity were obtained from interferograms taken from that region of the reconstruction. The background single-beam spectra used to calculate the percent reflectance spectra were the Fourier transforms of the interferograms taken outside of the two reconstruction peak areas. The substrate used was 600 grit (30 μm) diamond powder (Graves Company, Pompano Beach, FL). For all measurements, the optical system was not purged with nitrogen or dry air nor was a covering used to prevent air currents from disturbing the stability of the infrared source.

Continuous Collection Station. The interface designed for the collection and infrared analysis of compounds separated by a RP-μHPLC is shown in Figs. 2 and 3. The sample collection unit is made up of two components, the base and the translatable trough. The base, made from solid aluminum stock, is 30.5 cm long, 6.7 cm wide, and 1.8 cm thick. An H-shaped trench 29 cm long, 3.5 cm wide, and 1 cm deep was cut in the middle to accommodate the pulleys and the cable used for translating the movable trough.

The movable trough was also cut from a piece of solid aluminum stock. The trough cavity is 13 cm long, 7 cm wide, and 2 cm deep. The cavity opening is covered by a strip of coarse filter paper, which is held in place by a 1-mm-thick aluminum frame anchored to the perimeter of the cavity by ten 3-mm-long counter-sunk screws. The filter paper supports the diamond powder layer on which the analyte is deposited.

Inside the trough cavity is a directional vacuum port the opening of which is directed normal to and flush with the surface of the supporting filter paper. The vacuum port is a 4-mm-i.d. stainless-steel tubing with a 90° orifice extension made of Teflon®. The stainless-steel tubing passes through an O-ring-sealed hole on the wall cavity and is secured in a stationary position by set screws

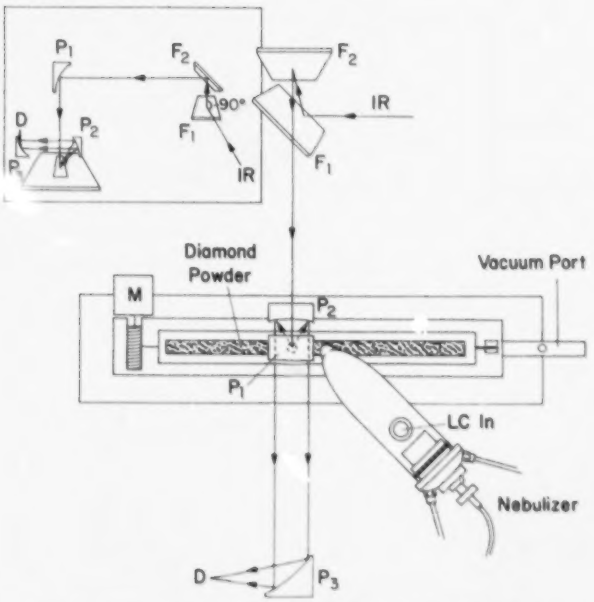

FIG. 3. Schematic diagram of RP-μHPLC/DRIFTS interface.

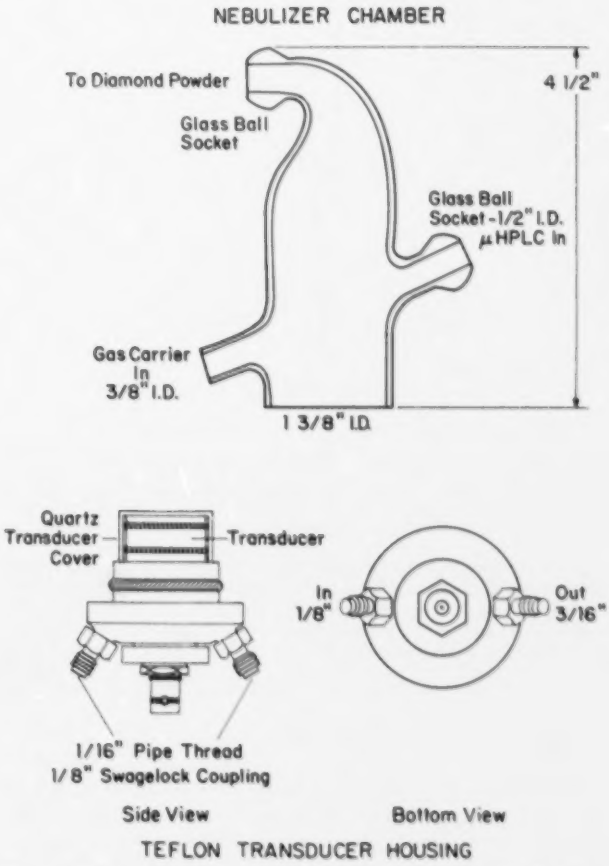

FIG. 4. Schematic drawing of the nebulizer chamber and transducer housing.

on a mounting flange of the base of the collection unit. The seal enables the maintenance of a pressure differential across the powder layer while the trough is translated during sample collection. A second vacuum port directly attached to the trough is installed to maintain a pressure differential sufficient to keep the diamond powder in a compact layer. The trough slides on a "dove tail" groove on the base of the collection unit.

Nebulizer Configuration. The nebulizer housing and chamber are shown in Fig. 4. The piezo-electric transducer was obtained from Channel Products (Chesterland, OH). The transducer was driven by a Plasma Therm power supply at an incident power of approximately 40 W at a frequency of 1.4 MHz. A constant flow of water at 3 to 5 gal/h was used to cool the transducer. The diameters of the water inlet and outlet ports were 4.8 mm and 3.2 mm, respectively. This configuration resulted in a slight backpressure on the cooling water, which helped prevent air bubbles from forming around the transducer. The air bubbles prevented the cooling water from coming in contact with the transducer, thereby causing hot spots to occur on the transducer surface. These hot spots eventually resulted in dead areas on the transducer surface.

The nebulizer chamber was constructed from a Pyrex® glass tube, 3.5 cm in diameter and approximately 15 cm long. The eluant from the RP-μHPLC was directed to the surface of the transducer through the LC-IN port of the chamber. The carrier gas used for purging the chamber was brought in through the CARRIER GAS-IN port. The gas flow used was approximately 1 L/min.

Eluate Collection Procedures. Both continuous and discrete peak collection procedures were used. When operating in the continuous collection mode, the trough is translated at a constant speed throughout the chromatographic separation process. Operationally, the chromatographic process, the GIFTS data collection program, and the trough translation were started at the same time. Approximately 25 interferograms were collected to form a basis set for the G.S. reconstruction. The basis set was collected while the trough was in motion in order to incorporate changes in surface reflectivity of the diamond powder into the basis set during data collection.

To conserve the length of the powder layer useful for sample deposition, the trough was repositioned to its initial starting position after the basis set was collected in some of the experiments. For the same reason, when the uv chromatographic detector is used, the trough translation, the GIFTS program, and the nebulization procedure are initiated only when the leading edge of the first eluting component is detected. When the uv detector is being bypassed, the chromatography is simultaneously initiated with the above three processes.

In experiments where the uv detector and the 50-cm transfer line are used, there is a lag of one minute, as the eluting zone travels from the uv detector to the nebulization chamber. Thus, discrete peak deposition is facilitated by stopping the trough translation one minute after the leading edge of the zone is detected and then restarting it after a period equivalent to the full width at half height of the eluting zone.

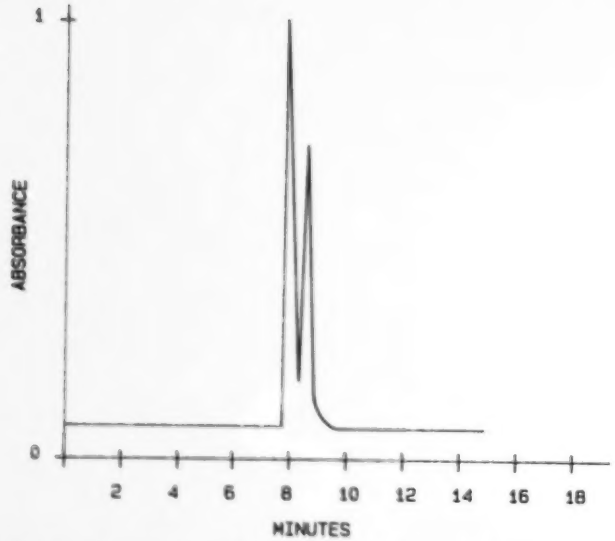

FIG. 5. Chromatogram of *m*-nitroaniline and 2-methyl-3-nitrophenol from the uv detector at 254 nm. The mobile-phase composition was 80:20 methanol/ water, flow rate was 40 μL/min with a 5-μm C_{18} microbore column and a 1-μL injection loop. Each peak corresponds to 3 μg (injected) of that component.

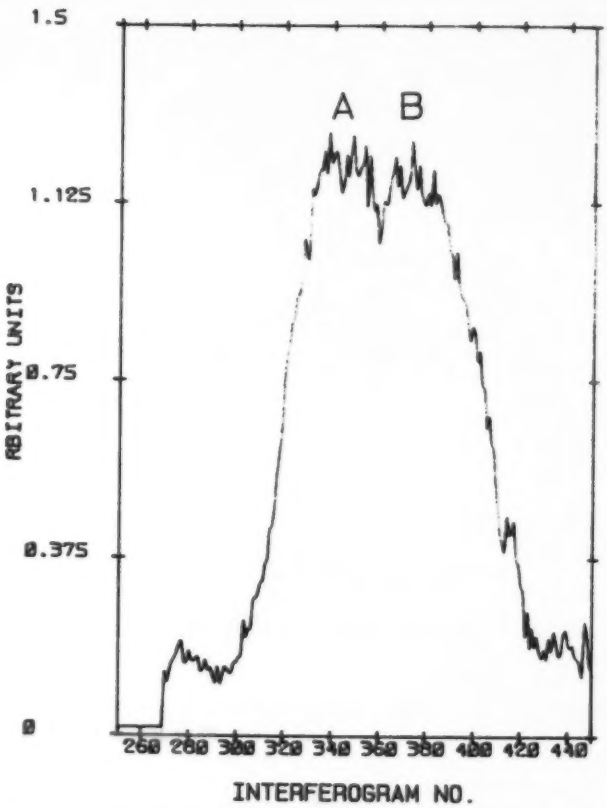

FIG. 6. Gram-Schmidt reconstruction of *m*-nitroaniline (A) and 2-methyl-3-nitrophenol (B) collected by the continuous collection procedure (trough translation rate of 4 mm/min).

The trough translation speeds depend primarily on the full width at half height (FWHH) of the sharpest peak in the chromatogram, the flow rate (FR), and the diameter of the deposition spot on the surface of the powder layer. The spot size is largely determined by the diameter (d) of the orifice of the directional vacuum port. It is sufficient to use Eq. 1,

$$S = d/(\text{FWHH})/\text{FR}, \quad (1)$$

to estimate the trough translation speed. In these experiments typical values of d are 3 mm or 5 mm, FWHH is 30 μL, and FR is 40 μL/min. Two values for S, 4 mm/min and 7 mm/min, were used for the data collection.

RESULTS AND DISCUSSION

The collection station (Fig. 2) incorporates the essential features of a truly versatile on-line HPLC/FT-IR interface. It permits the collection of diffuse reflectance spectra during a continuous chromatographic elution process. It yields a real-time reconstruction of the chromatogram for both continuous and discrete sampling collection modes.

No post-column isolation of the analyte from the reverse-phase solvent is needed. This is made possible primarily by two factors: the use of diamond powder, which has comparable diffuse reflectance characteristics to those of KCl but is inert with respect to most reverse-phase solvents, and the use of the nebulizer, which not only facilitates the rapid desolvation of the aerosols but also keeps the diamond powder layer undisturbed during the process of sample deposition.

A separate aerosol desolvation chamber was not necessary for the microbore HPLC process. It appears that, for microbore HPLC, the volumetric flow rates are sufficiently low for the efficient generation of aerosol droplets, which are small enough to permit desolvation of the solute before reaching the powder layer.

The piezo-electric nebulizer, however, does not produce uniformly sized droplets. Occasionally, large droplets that tend to wet and disturb the powder surface will be produced. The nebulizing surface is also not uniformly effective and tends to aggravate the large droplet formation.

The vertical orientation of the nebulizing surface appears to help keep most of the large droplets from reaching the powder layer. However, the introduction of the purge gas into the chamber probably minimizes the benefit of the nebulizer orientation. It was considered more important to minimize zone mixing of the separated components in the nebulization chamber; hence the necessity for a purge gas.

Although wetting of the powder surface may occur occasionally during the on-line run, there is no need to halt the analysis. Analysis may continue until the chromatography is completed. The trough is then reset to its initial position and the GIFTS program is rerun at the same trough translation speed. The vacuum in the trough cavity seems to aid in the rapid removal of any remaining solvent. Diffuse reflectance spectra obtained from such a rerun of the GIFTS program were free of solvent absorption.

The partial vacuum in the trough cavity not only aids in the completion of desolvation but also serves to main-

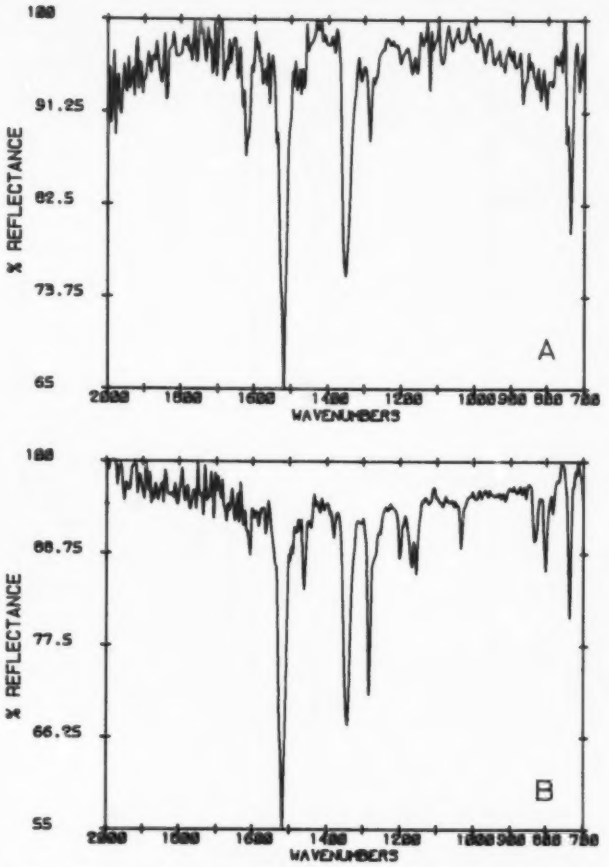

FIG. 7. Spectra of *m*-nitroaniline (A) and 2-methyl-3-nitrophenol (B), 3 μg of each injected, collected by the continuous collection procedure (trough translation of 4 mm/min). Interferogram no. 350 was used for sample spectrum A and no. 374 was used for sample spectrum B.

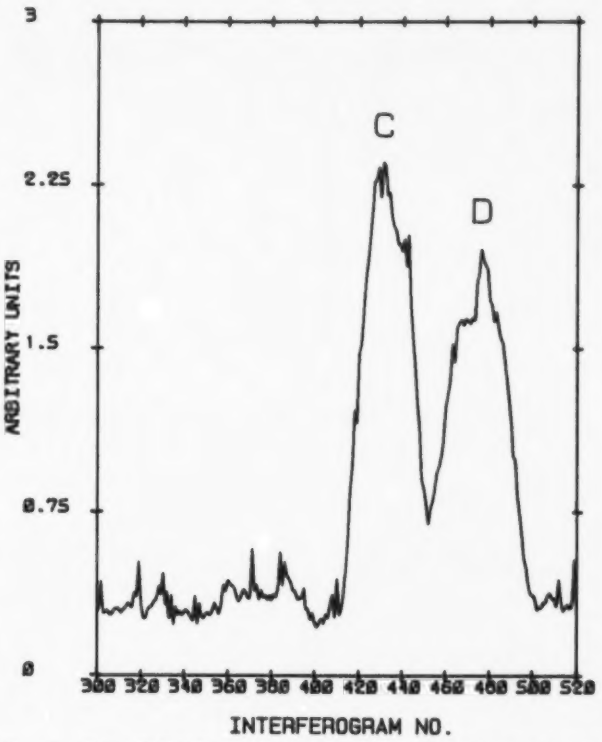

FIG. 8. Gram-Schmidt reconstruction of *m*-nitroaniline (C) and 2-methyl-3-nitrophenol (D) collected by the continuous collection procedure (trough translation speed of 7 mm/min). The uv detector cell was bypassed.

tain the entire powder layer in a useful degree of compactness. Of equal significance is the use of the directional vacuum port, which enables the nebulized material to be deposited uniformly on a small circular area at the surface of the powder layer. By trying different diameters for the orifice, one can obtain a spot size of roughly the same diameter as the focus of the incident beam on the powder layer, thereby optimizing the sensitivity of the interface.

These observations indicate that it may be feasible to couple the collection station with a normal-bore RP-HPLC column if an aerosol desolvation section is inserted between the nebulization chamber and the powder layer. The problem of nonuniform droplet formation may be minimized by using a monodispersed aerosol generator[13] instead of the piezo-electric nebulizer.

Disturbance in the recorded interferogram may arise not only from the disturbance of the powder layer during sample deposition but also from the jerkiness of the translation of the trough during an on-line run. The best situation would have been to hold the trough stationary during the active scan of the interferometer and then translate the trough during retrace. This, however, fixed the trough translation speed, thus preventing versatile adaptation to various rates of sample deposition as dictated by the chromatographic process. A useful and effective compromise is to translate the trough smoothly at a constant speed. This is accomplished in the current device by means of a cable and pulley system.

The pulley and cable drive helped to absorb the sudden "jerk" that resulted each time the motor was stepped. Consequently, the trough moved at almost a constant rate, rather than in discrete steps. This continuous motion of the trough resulted in a more stable interferogram and, thus, a lesser possibility of introducing spectral artifacts.

To evaluate the effectiveness of the interface, analysis of a test sample was conducted. A solution containing 3 μg/μL of *m*-nitroaniline and 2-methyl-3-nitrophenol, a 1-mm-i.d. C_{18} column, a 1-μL injection loop, a mobile-phase composition of 80:20 methanol/water, and a uv detector (254 nm) were used in all the test runs. The partially resolved components due to the mobile-phase composition are shown in the chromatogram in Fig. 5. The partially resolved zones enabled us to evaluate how effectively the interface preserves the integrity of the chromatographic resolution.

Severe problems due to post-column band broadening were encountered in the continuous on-line mode of analysis.[14,15] With the uv detector cell and a 50-cm flexible silica extension tube in the flow path, no useful G.S.-reconstructed chromatogram was obtained at the translation speed of 7 mm/min. With the use of exactly the same conditions but at a reduced trough translation speed of 4 mm/min, the G.S.-reconstructed chromato-

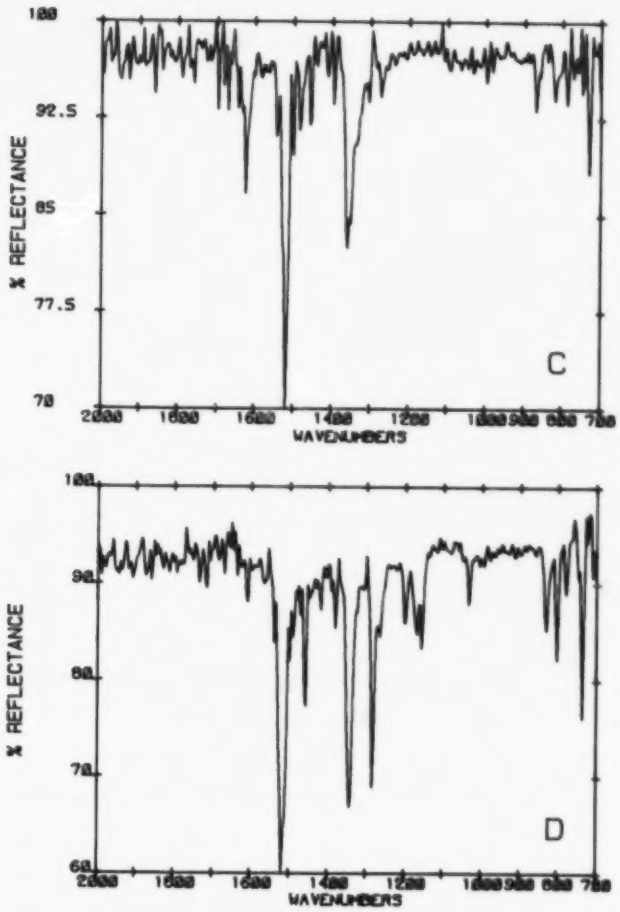

FIG. 9. Spectra of *m*-nitroaniline (C) and 2-methyl-3-nitrophenol (D), 3 μg of each injected, collected by the continuous collection procedure (trough translation of 7 mm/min, uv detector cell bypassed). Interferogram no. 420 was used for sample spectrum C, and no. 471 was used for sample D.

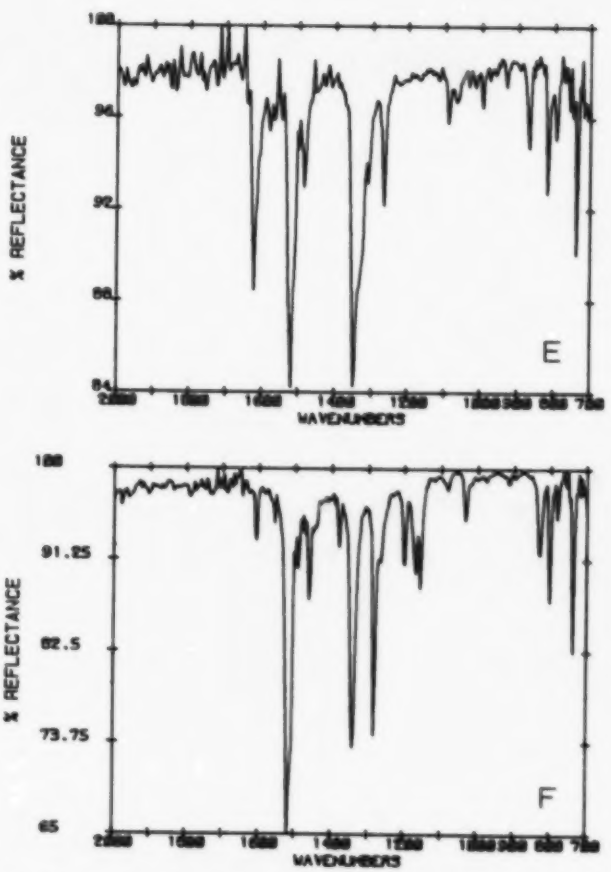

FIG. 10. Spectra of *m*-nitroaniline (E) and 2-methyl-3-nitrophenol (F) obtained from 16 coadded, double-precision interferograms.

gram shown in Fig. 6 was obtained. The poor signal-to-noise (S/N) ratio and substantially reduced resolution of the reconstructed chromatogram indicate the extensive degree of dilution and mixing as the eluted zones move through the uv detector cell and the extension tube. The reconstructed chromatogram contains the two components of the test solution. This is verified by spectra shown in Figs. 7A and 7B, which were derived from the Fourier transform of the interferograms in regions 6A and 6B, respectively. Both spectra are of sufficient S/N for a qualitative analysis of the separated compounds.

By the use of a short (10-cm-long, 0.2-mm-i.d., flexible silica) transfer tube directly connected to the output end of the column and bypassing the uv cell, the G.S.-reconstructed chromatogram shown in Fig. 8 was obtained at a trough translation speed of 7 mm/min. It is easy to see that the resolution of the components in the G.S.-reconstructed chromatogram is not too different from that of the chromatogram in Fig. 5. The relative intensities of the peaks in Figs. 5 and 8 are not the same. This is not unexpected, however, in view of the different bases and principles involved in the construction of the two chromatograms. The spectra of each of the test compounds obtained from the Fourier transform of the interferograms from peaks 8C and 8D are shown in Figs. 9C and 9D, respectively.

Considerable improvement of the signal-to-noise ratio of the spectra of the deposited substances can be obtained by running the sequential, double-precision, coadded, scan-set mode of disk data collection over the region of deposition at the same trough translation speed that was used for the on-line run. The spectra of the two components obtained in this manner showing the improved signal-to-noise ratio by the signal coaddition processes are shown in Fig. 10. With the availability of faster scanning interferometers, this would be the most efficient mode for on-line, real-time data collection and analysis. The time, t_s, for a scan set can be adjusted so that the minimum number of points, n_s, required to define the profile of the sharpest peak may be calculated from

$$n_s = d/S/t_s \qquad (2)$$

where d and S are as defined previously. A value of n_s of at least 3 may suffice for the chromatographic reconstruction process.

The interface may also be used for discrete sample

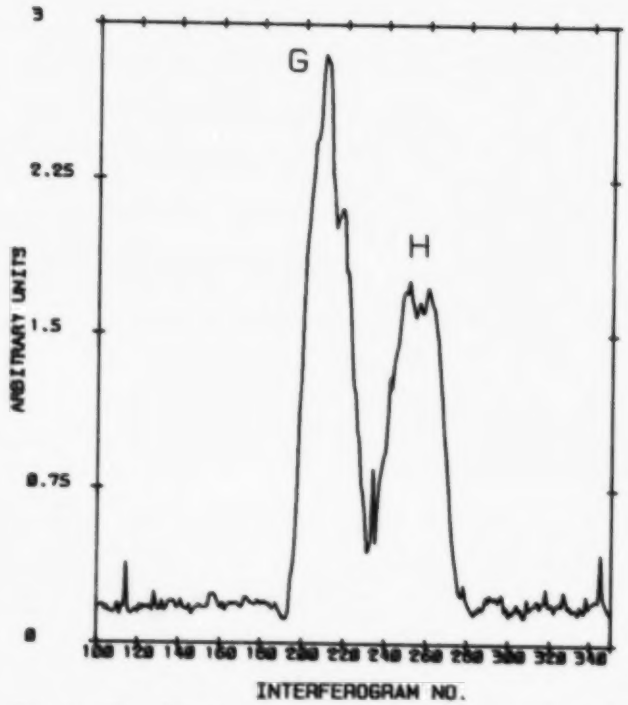

FIG. 11. Gram-Schmidt reconstruction of *m*-nitroaniline (G) and 2-methyl-3-nitrophenol (H) collected onto the diamond powder with the discrete sample collection procedure.

collection. This capability would be most useful if one is interested primarily in a specific number of eluted zones in the chromatograms; if one needs to accumulate a sufficient amount of the analyte on a small area of the diamond powder layer by multiple runs of the sample; or because the eluting zone is so broad that the trough translation speed calculated for the optimum resolution of the sharpest peak in the chromatogram will result in spreading the eluting zone over a large area such that no useful spectrum of the substance can be obtained.

The results of our attempts to simulate the latter case, where the two zones were broadened and partially mixed while passing through the uv detector and the 50-cm transfer line, are shown in Fig. 11. In this process one simply stops the moving trough when the zone starts to elute, taking the delay time into account. The delay time is simply the time required for the eluted zone leaving the column to reach the nebulizing chamber. The trough remains stationary only for a period corresponding to the FWHH of the zone. The two components of the test solution were isolated in this manner under adverse conditions of peak broadening and partial mixing, as indicated by the G.S.-reconstructed chromatogram in Fig. 6. Nevertheless, a relatively pure spectrum of each of the components was obtained. These spectra are shown in Fig. 12.

CONCLUSION

A versatile on-line reverse-phase HPLC/FT-IR interface that facilitates the continuous collection of diffuse reflectance interferograms during the chromatographic

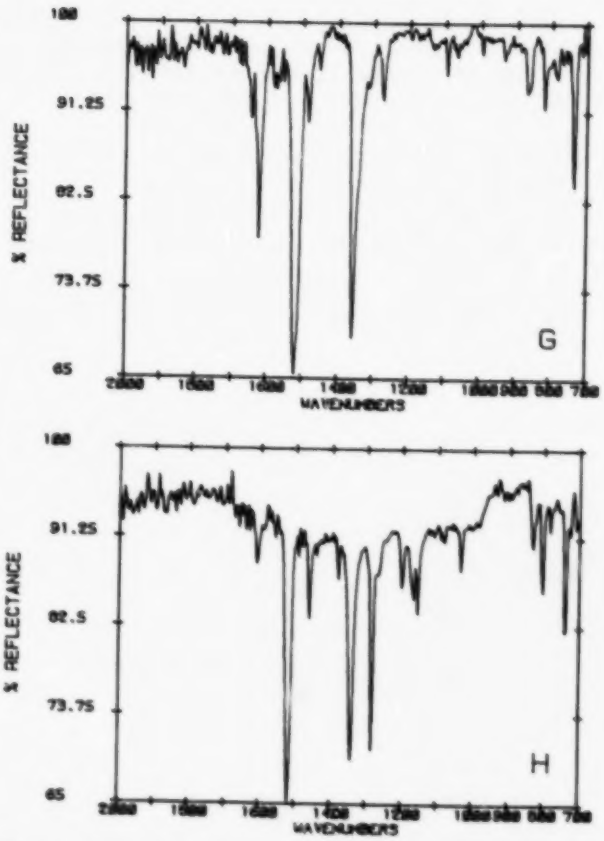

FIG. 12. Spectra of *m*-nitroanaline (G) and 2-methyl-3-nitrophenol (H), 3 μg of each injected, collected from the RP-μHPLC by the discrete collection procedure. Both spectra are 8 cm^{-1} resolution and single scan, single precision. Spectrum G was obtained from interferogram no. 207, and spectrum H was obtained from interferogram no. 258 in the reconstruction. Both spectra used references in the interferogram no. 150 range of the reconstruction.

elution process has been successfully developed. No post-column chemical treatment for the isolation of the chromatographically separated sample components is needed to obtain their respective diffuse reflectance spectra. The chromatographic resolution is preserved during the on-line deposition process. The diamond powder used as the diffuse reflectance matrix may be cleaned and reused almost indefinitely.

The detection limit of the current interface is relatively high, but this was not totally unexpected. A more efficient diffuse reflectance collection optics and a purged and enclosed system to exclude atmospheric CO_2 water vapor, as well as the vaporized mobile-phase solvent from the optical path, coupled with the double-precision coadded scan-set mode of data collection could dramatically improve its sensitivity.

On the other hand, the high detection limit could be due to the deposition of microcrystals formed from the desolvated aerosols. A molecular deposit on the surface of the diffuse reflectance matrix would be most desirable, and this may be achieved by controlling the size of the aerosol droplets so that they are not completely desolvated when these are deposited on the powder surface.

Although additional work is needed to improve the sensitivity, the major problems associated with the development of a viable on-line reverse-phase HPLC/FT-IR interface appear to have been resolved.

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